Toxicology in Vitro 29 (2015) 1729–1735

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Effects of selective serotonin reuptake inhibitors on three sex steroids in two versions of the aromatase enzyme inhibition assay and in the H295R cell assay

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ARTICLE INFO

Article history:
Received 16 February 2015
Revised 2 July 2015
Accepted 6 July 2015
Available online 7 July 2015

Keywords:
Citalopram
Endocrine disruption
17β-estradiol
Fluoxetine
Fluvoxamine
Paroxetine
Progesterone
Sertraline
SSRI
Steroidogenesis
Testosterone

ABSTRACT

Selective serotonin reuptake inhibitors are known to have a range of disorders that are often linked to the endocrine system e.g. hormonal imbalances, breast enlargement, sexual dysfunction, and menstrual cycle disorders. The mechanisms behind most of these disorders are not known in details. In this study we investigated whether the endocrine effect due to SSRI exposure could be detected in well adopted in vitro steroidogenesis assays, two versions of the aromatase enzyme inhibition assay and the H295R cell assay. The five drugs citalopram, fluoxetine, fluvoxamine, paroxetine and sertraline, were shown to inhibit the aromatase enzyme in both types of aromatase assays. The IC_{50} values ranged from 3 to 600 μM. All five SSRIs, were further investigated in the H295R cell line. All compounds altered the steroid secretion from the cells, the lowest observed effect levels were 0.9 μM and 3.1 μM for sertraline and fluvoxamine, respectively. In general the H295R cell assay was more sensitive to SSRI exposure than the two aromatase assays, up to 20 times more sensitive. This indicates that the H295R cell line is a better tool for screening endocrine disrupting effects. Our findings show that the endocrine effects of SSRIs may, at least in part, be due to interference with the steroidogenesis.

1. Introduction

Selective serotonin reuptake inhibitors (SSRIs) are a group of drugs that are highly used to treat depression as well as a range of other psychiatric disorders e.g. anxiety and eating disorders in both adults and children (Vaswani et al., 2003). Citalopram (and its S-stereoisomer escitalopram), fluoxetine, fluvoxamine, paroxetine and sertraline all belong to this group of drugs. The selected SSRIs are among the most applied worldwide. Since the SSRIs were first marketed, starting with fluoxetine, the use of the SSRIs has increased steadily. The total use of SSRIs in Denmark in dosages per year has increased from 17 million in 1994 to 102 million in 2013 (Danish Medicines Agency, 2004, 2010). As reviewed by Kreke and Dietrich, similar sales trends have been observed in other western countries (Kreke and Dietrich, 2008).

Literature reports that many of the adverse effects of SSRIs may be connected to the endocrine system. Some examples are reported below. Sexual disorders are very common in SSRI users, as 30–60% of all SSRI users have been reported to suffer from sexual dysfunction in some form (Gregorian et al., 2002). In adult males it has been shown that SSRI use is associated with hormonal abnormalities compared to healthy individuals. This trend is even more pronounced when sexual dysfunction is present. The hormonal abnormalities were lowered levels of testosterone (TS), luteinizing hormone (LH), and follicle stimulating hormone (FSH), and elevated level of prolactin (Safarinejad, 2008). In a study on young men, fluoxetine treatment altered the individual (TS) level, however the effects seen were both increases and decreases and overall the effect on the (TS) level was cancelled out (Bell et al., 2006). In adult females SSRI use has been associated with breast enlargement and change of the menstrual cycle length (Amsterdam et al., 1997; Steiner et al., 1997).

Abbreviations: d7-AN, D7-androstenedione; AN, androstenedione; CYP19, cytochrome-P450 family 19/aromatase enzyme; E2, 17β-estradiol; H295R, human adrenocortical carcinoma cell line; SSRI, selective serotonin reuptake inhibitor; TS, testosterone; PRO, progesterone.

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http://dx.doi.org/10.1016/j.tiv.2015.07.005
0887-2333/

In female rats fluoxetine has been shown to influence the estrous cycle, sexual behaviour, progesterone (PRO) levels and 17β-estradiol (E2) levels (Matuszczyk et al., 1998; Taylor et al., 2004; Uphouse et al., 2006). Also, in other vertebrates such as fish, fluoxetine has been shown to influence E2 levels (Foran et al., 2004; Lister et al., 2009; Mennigen et al., 2008). Fluvoxamine has also been shown to influence the circulating E2 and PRO levels in female rats (Rehavi et al., 2000). Furthermore the compounds have been shown to have detrimental effects on bone in rats and mice (Bonnet et al., 2007; Warden et al., 2008; Westbroek et al., 2007).

The described adverse effects may be due, at least in part, to a direct effect of the SSRI on the steroidogenesis. Aromatase catalyses the aromatisation of the A ring and the cleavage of the methyl group at carbon 10 of the steroid ring system of androstenedione (AN) and (TS) to produce estrone and (E2), respectively, as presented in Fig. 1 (Simpson et al., 1994). Interference with the aromatase enzyme can be investigated in different well-established in vitro assays using e.g. microsomes from insect cells transfected with human CYP19 (aromatase enzyme) as the enzyme source, or using more complex systems such as the H295R cell line. The H295R cell is an adrenergic carcinoma cell line which expresses all the enzymes in the steroidogenesis and has previously been used to study endocrine disrupting effects of drugs (Nielsen et al., 2012; Winther et al., 2013). Both methods have been utilized in the present work. Microsomes offer a direct way of detecting interferences with the enzyme, as very few other enzymes are present, and there are no cell membranes that need to be passed by the substrates or test compound. The microsomes can be used with different substrates; both artificial and natural (Brueggemeier et al., 2005). OECD (2011) has recently published a guideline for screening chemicals using the H295R cell line.

Although we might expect some effects on sex hormones due to interactions with transmitter control mechanisms, we wanted to determine whether these compounds might have effect on the enzymes producing the hormones directly.

In the present study we therefore explored whether the selected steroidogenesis in vitro assays were able to demonstrate interference with the hormone system due to SSRI exposure. First, the compounds were tested in two similar microsome based aromatase assays, one with an artificial substrate (fluorescence detection assay), and one with a natural substrate (GC–MS quantification assay). Secondly, the compounds were selected and tested in the more complex test system, the H295R cell line. Finally, the results obtained in the three assays were compared.

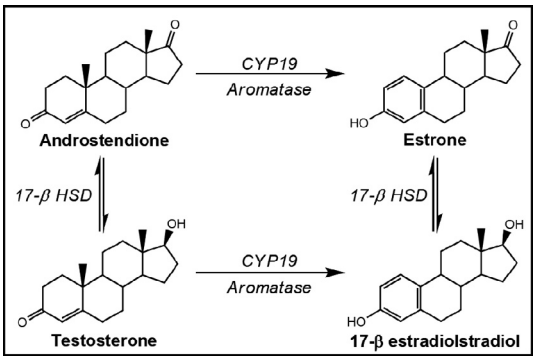

Fig. 1. The structures and enzymatic conversions between androgens and estrogens are shown. Aromatase (CYP19) is the enzyme that converts androgens (testosterone and androstenedione) to estrogens (estrone and estradiol). During the enzymatic conversion a methyl group is removed and an aromatic ring is formed. In the figure the following abbreviations are used: cytochrome P450 (CYP), hydroxysteroid dehydrogenase (HSD).

2. Experimental procedures

2.1. Chemicals, enzymes and cells

The test compounds fluvoxamine maleate (CAS # 61718-82-9), fluoxetine hydrochloride (CAS # 59333-67-4), sertraline hydrochloride (CAS # 79617-96-2), paroxetine hydrochloride (CAS # 61869-08-7) and citalopram hydrochloride (CAS # 85118-27-0) were obtained from TRC (North York, ON, Canada). Androstenedione (CAS # 63-05-8) and methylthiazolyldiphenyl-tetrazolium bromide (MTT; CAS # 298-93-1) were obtained from Sigma–Aldrich (Steinheim, Germany). D7-androstenedione (d7-AN) (CAS # 67034-85-9) was purchased from CDN-isotopes (Essex, United Kingdom). Dibenzylflourescein (DBF; CAS # 97744-4-0) was purchased from BD Gentest (Woburn, MA, USA). Stock solutions of the above mentioned test compounds and chemicals were prepared in methanol and stored at −25 °C.

Acetonitrile (CAS # 75-05-8), methanol (CAS # 67-56-1), heptane (CAS # 142-82-5) and isopropanol (CAS # 67-63-0), all HPLC-grade, were all obtained from LabScan (Dublin, Ireland). All water was purified with a MilliQ water apparatus. Hydrochloric acid (HCl; CAS # 7647-01-0) and sodium hydroxide (NaOH; CAS # 1310-73-2) were purchased from Riedel-de-Häen (Seelze, Germany). Magnesium chloride hexahydrate (CAS # 7791-18-6) and potassium dihydrogen phosphate (CAS # 7778-77-0) were purchased from Merck (Darmstadt, Germany).

The human aromatase enzyme (CYP19) co-expressed with NADPH cytochrome P450 reductase supersomes (catalogue # 456260) and control supersomes (catalogue # 456201) were obtained from BD Gentest (Woburn, MA, USA). NADPH (CAS # 2646-71-1) was from AppliChem GmbH (Darmstadt, Germany). The H295R human adrenocortical carcinoma cell line was from American Type Culture Collection (ATCC, #CRL-2128, Manasses, VA, USA). DMEM/F12 medium from (GibcoBRL Life Technologies, Paisar, UK). ITS + Premix and Nu-serum were from BD Bioscience (Brøndby, Denmark).

2.2. Aromatase assay using fluorescence detection for quantification

The determination of aromatase inhibition with fluorescence detection for quantification of processes developed by Stresser et al. (2000) was performed as described in a previous publication (Jacobsen et al., 2008). In brief: the test compounds were incubated with 2 nM aromatase supersomes, 250 μM NADPH, and the 0.4 μM artificial substrate DBF. During the incubation with aromatase and the subsequent alkaline post incubation, DBF was converted to fluorescein. The relative amount of fluorescein corresponds to the aromatase activity. The fluorescence was measured on a Wallac EnVision™ Xcite™ (Perkin Elmer, Turku, Finland) at excitation wavelength 495 nm and emission wavelength 535 nm.

2.3. Aromatase assay using quantification with GC–MS/MS

The test compounds were initially diluted with buffer (50 mM potassium phosphate buffer (pH 7.4) containing 0.5 mM magnesium chloride). The compounds were tested at eight logarithmically spaced concentrations with each concentration analysed in triplicate. All five SSRIs were tested three times.

100 μl of the test compound solutions were deposited in reaction tubes and the closed reaction tubes were heated to 37 °C in an incubation oven. All other incubations in the assay were performed under the same conditions. 50 μl buffer containing 8 nM aromatase enzyme and 1.8 μM AN was added and the mixtures were preincubated for 10 min. The enzymatic reaction was initiated by adding 50 μl buffer with 1 mM NADPH, and the reaction